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14. ABSTRACT

Genotoxicity is often linked to carcinogenesis. The assessment of the genotoxic potential of any "agent" is important in terms of suggesting its carcinogenic potential. Hence, the evaluation of the genotoxic potential of jet fuels, JP-8 and Jet-A has been proposed in these investigations. The rodent micronucleus (MN) assay, which detects micronuclei, arising from both chromosomal fragments and unequal segregation of chromosomes during cell division has been widely applied as an in vivo assay for detecting genotoxic agents. The assay became a standard test system for genotoxicity evaluations in regulatory agencies in several countries (Sofuni 1993, Kirkland 1993, Auletta et al., 1993, Health Protection Branch Genotoxicity Committee, Canada, 1993).

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B. Manuscripts

(1) "Micronucleus Studies in the Peripheral Blood and Bone Marrow of Mice Treated with Jet Fuels, JP-8 and Jet-A" by Vijayalaxmi, Andrew D. Kligerman, Thomas J. Prihoda and Stephen E. Ullrich. Cytogenetics and Genome Research. **104**, 371-375, 2004.

(2) "Micronucleus Studies in the Peripheral Blood and Bone Marrow of Mice Treated with Jet Fuels, JP-8 and Jet-A" by Vijayalaxmi, Andrew D. Kligerman, Thomas J. Prihoda and Stephen E. Ullrich. Mutation Research. **608**, 82-87, 2006

(3) "Cytogenetic Studies in Human Blood Lymphocytes Exposed *In Vitro* to 2.45 GHz or 8.2 GHz Radiofrequency Radiation" by Vijayalaxmi. Radiation Research. **166**, 532-538, 2006.

C. Scientific Personnel Supported by this grant

Vijayalaxmi, Ph.D., Principal Investigator, 100% time
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D. Inventions/Patents/Discoveries

None

E. Collaborators

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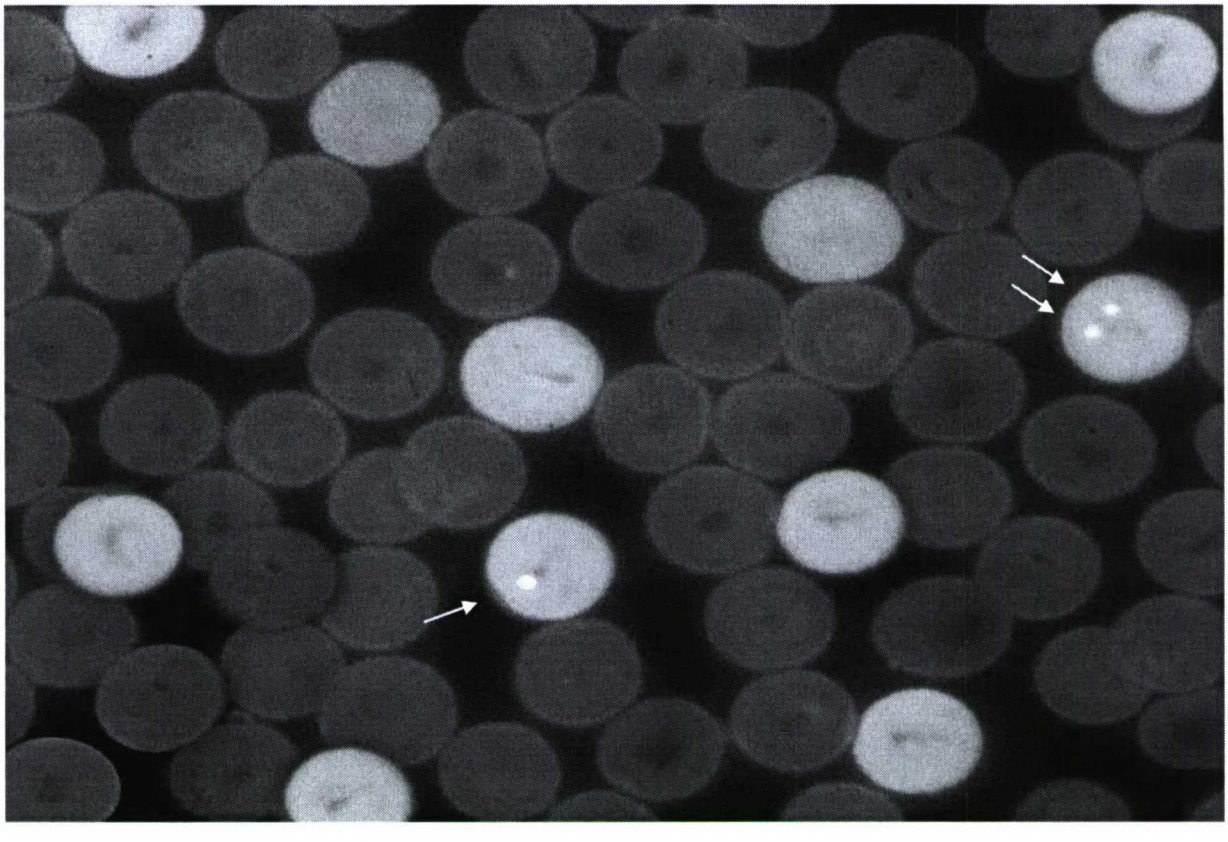
None

G. Key Findings/Results/Accomplishments

INTRODUCTION

Genotoxicity is often linked to carcinogenesis. The assessment of the genotoxic potential of any "agent" is important in terms of suggesting its carcinogenic potential. Hence, the evaluation of the genotoxic potential of jet fuels, JP-8 and Jet-A has been proposed in these investigations. The rodent micronucleus (MN) assay, which detects micronuclei, arising from both chromosomal fragments and unequal segregation of chromosomes during cell division has been widely applied as an *in vivo* assay for detecting genotoxic agents. The assay became a standard test system for genotoxicity evaluations in regulatory agencies in several countries (Sofuni 1993, Kirkland 1993, Auletta et al., 1993, Health Protection Branch Genotoxicity Committee, Canada, 1993). A photomicrograph of polychromatic erythrocytes (PCE, immature erythrocytes) containing micronuclei (MN, indicated by arrows) in mouse blood is presented below.



MATERIALS AND METHODS

Mice were used as experimental animals for all of the proposed investigations. JP-8 and Jet-A were supplied by The Operational Toxicology Branch, Air Force Research Laboratory, Wright Patterson Air Force Base, Dayton, OH. Both fuels were stored and used in a chemical fume hood.

(1) Dermal exposures: These studies were conducted in collaboration with Dr. Stephen E. Ullrich at the Department of Immunology, University of Texas MD Anderson Cancer Center in Houston, Texas. Experiments were conducted using pathogen-free adult, 8 to 10 weeks old, C3H/HeNCr (MTV-) female mice. These mice were obtained from the National Cancer Institute Frederick Cancer Research Facility Animal Production Area (Frederick, MD). The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and National Institutes of Health and the National Toxicology Program. All animal handling procedures were reviewed and approved by the University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee.

Two hours before the start of all experiments, an area of approximately 8 cm^2 on the backs of the mice was shaved. Animals which received no further treatment were used as controls. Other mice were treated with 300 µl (or 240 mg either a single or increasing doses) of JP-8 or Jet-A: this dose was chosen because, in previous studies, application of 300 µl of JP-8 and Jet-A was found to be immunotoxic (Ullrich, 1999; Ramos et al., 2002). The undiluted fuel (300 µl) was applied directly to the shaved dorsal skin with a micropipette. The mice were then caged individually in the chemical fume hood for the next 3 hours. This prevented cage mates from grooming and ingesting the fuel. After 3 hours the residual fuel was either absorbed through the skin of the mice or had evaporated. The animals were then returned to standard housing in an SPF-barrier facility. In all experiments, mice which were injected with cyclophosphamide (CP, dissolved in sterile phosphate buffered saline, intra-peritoneal injection to give a final dose of 40 mg/kg body weight) were used as positive controls.

(2) Inhalation exposures: These studies were conducted in collaboration with Dr. Mark L. Witten at the Department of Pediatrics, University of Arizona Health Science Center in Tucson, Arizona. Experiments were conducted using adult, 8 to 10 weeks old, male Swiss-Webster mice. Aerosolized JP-8, 50 mg/m^3 and 70 mg/m^3, was used to expose the mice for 1 hour/day for 7 consecutive days. Mice which were kept in laboratory cages were used as controls. In all experiments, mice which were injected with mitomycin C (MMC, dissolved in sterile phosphate buffered saline, intra-peritoneal injection to give a final dose of 1 mg/kg body weight) were used as positive controls.

From each animal, blood and bone marrow smears were prepared as described below.

Blood: At the time of necropsy, blood was collected by heart puncture using a 1 ml syringe fitted with a 22G needle (the tip of the tail was cut with a pair of sharp scissors to obtain a small drop of blood, from the same animal, for serial examinations/assessments). Immediately, a small drop of blood was placed on 2-4 clean microscopic slides and pushed behind another slide held at a 45° angle to form a thin smear over an area of 3-4 cm. All smears were air-dried for 30-45 minutes. The cells on the slides were then fixed in absolute methanol for 30 minutes. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C.

Bone marrow: At the time of necropsy, from each animal, both femur bones were collected and the bones were cleaned of the surrounding muscle tissue. A 22 G needle fitted to a syringe filled with 1 ml of fetal calf/bovine serum (heat inactivated at 56°C for 2 hours) was inserted at one end of the bone to flush the marrow into a 1.5 ml microfuge tube. The marrow (in the tube) was then pulled gently up and down in the syringe, until a fine cell suspension was observed in the serum. All tubes were gently centrifuged for few seconds to pellet the cells at the bottom of the tube. Most of the supernatant was discarded, and the cells in the pellet were re-suspended in a very small volume of the remaining serum. Small drops of the resulting viscous cell suspension were placed on 2-4 clean microscope slides, and thin smears were made as described for the blood. All smears were air-dried for 30-45 minutes. The cells on the slides were then fixed in absolute methanol for 30 minutes. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C.

Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium phosphate buffer, pH 7.4) before microscopic examination. In some experiments, in addition to Dr. Vijayalaxmi (investigator-1), Dr. Andrew Kligerman (investigator-2) at the Environmental Carcinogenesis Division, U.S. Environmental Protection Agency (EPA), Research Triangle Park, NC, has examined a complete and duplicate set of 'coded' microscope slides prepared from the blood and bone marrow smears from the mice. This step was initiated to obtain an independent evaluation and confirmation of the observations.

Data Collection: All slides were examined under 1000x magnification using a fluorescence microscope fitted with appropriate filters for acridine orange stain. For each animal, 2000 consecutive polychromatic erythrocytes (PCE, immature erythrocytes) were examined in the blood and bone marrow to determine the incidence of MN. In some experiments, for each animal, 10000 consecutive normochromatic erythrocytes (NCE, mature erythrocytes) in the blood and 2000 consecutive NCE in the bone marrow were examined to determine the incidence of MN. The results were decoded after microscopic evaluation was completed. The data were then subjected to statistical analyses.

Statistical Analyses: A SAS User's Guide was used for statistical analyses. The data were subjected to the analysis of variance (ANOVA) test to assess significant differences between groups, tissues collection times and doses of jet fuels. Treatment means were compared with the concurrent controls using a one-tailed Dunnett's test. Multiple as well as pair-wise comparisons were also made. The data were also analyzed for investigator variability. Pooled data was considered only when there were no significant differences between the two investigators. Square root transformation of the data was used when the conditions for such analyses were valid. Residuals were analyzed for homogeneity of variance and normality of distributions. Statistical significance was taken at a level of $p < 0.05$.

RESULTS

DERMAL EXPOSURE

Collaboration Studies with Dr. Stephen E. Ullrich in HOUSTON

Experiment #1: Preliminary Study 1

Aim: To determine the incidence of MN in the blood and bone marrow cells of mice exposed to JP-8 (dermal) – Evaluation of genotoxicity in PCE.

The protocol used for the experiment is as follows. A total of 15 adult, 8 to 10 weeks old, C3H/HeN (MTV-) female mice were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls, shave only - 5 mice
Group 2: JP-8, 300 µl applied on the skin - 5 mice
Group 3: UV-B radiation, 15 J/m^2 (positive controls) - 5 mice
Blood smears were prepared at 24 hours after JP-8 application.
Bone marrow smears were prepared at 24 hours after JP-8 application.

Two hours before the start of the experiment, an approximately 8 cm^2 area on the backs of the mice was shaved. Mice in group 1 received no further treatment and were used as controls. Mice in group 2 were treated with a single dose of 300 µl of JP-8. Mice in group 3 were exposed to 15 J/m^2 UV-B radiation and were used as positive controls. All mice were sacrificed at 24 hours after JP-8 treatment. . Blood and bone marrow samples were collected and smears were prepared on clean microscopic slides. All smears were air-dried and the cells on the slides were fixed in absolute methanol. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C. Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium phosphate buffer, pH 7.4) before microscopic examination. For each animal, 2000 consecutive PCE in blood and bone marrow were examined to determine the incidence of MN. The results were decoded after complete microscopic evaluation.

Results: The incidence of MN/2000 PCE presented in **Table H-1** indicated a small increase in the frequency of MN in both blood and bone marrow cells of mice exposed to JP-8 as compared with control mice. The positive control mice injected CP showed the expected significant increases in MN in both tissues.

Table H-1 (Experiment 1 in Houston): Incidence of micronuclei (MN in PCE) in the blood and bone marrow of mice exposed to 300 µl JP-8 (dermal).									
Group	# Mice Studied	Total PCE Examined	Individual Mouse Data MN/2,000 PCE					Group Mean MN in 2,000 PCE	Std. Dev.
BLOOD – PCE									
Control	5	10,000	4	5	3	4	4	4.0	0.4
JP-8	5	10,000	6	8	4	4	6	5.6	0.8
UV-B	5	10,000	8	5	6	6	6	6.2	0.5
BONE MARROW – PCE									
Control	5	10,000	8	8	9	7	9	8.2	0.4
JP-8	5	10,000	10	11	9	12	8	10.0	0.8
UV-B	5	10,000	12	11	10	10	9	10.4	0.6
Blood and bone marrow were collected when the mice were sacrificed at 24 hours after JP-8 treatment. In each mouse, 2000 consecutive PCE in the blood and bone marrow were examined. UV-B (ultra-violet radiation): 15 kJ/m^2.									

Experiment #2: Preliminary study 2

Aim: To determine the incidence of MN in the blood and bone marrow cells of mice exposed to JP-8 and Jet-A (dermal) – Exposure-Time-Course evaluation of genotoxicity in PCE.

The results from the first preliminary experiment indicated an increased incidence of MN in both the blood and bone marrow cells of mice exposed to JP-8. A repeat experiment was conducted using jet fuels, JP-8 and Jet-A.

The protocol used for the experiment is as follows. A total of 20 adult, 8 to 10 weeks old, C3H/HeN (MTV-) female mice were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls, shave only - 2 mice
Group 2: JP-8, 300 µl applied on the skin - 6 mice
Group 3: Jet-A, 300 µl applied on the skin - 6 mice
Group 4: CP-40 mg/kg body weight (positive controls) - 6 mice
Blood smears were prepared at 24, 48 and 72 hours after jet fuel application.
Bone marrow smears were prepared at 24, 48 and 72 hours after jet fuel application.

Two hours before the start of the experiment, an approximately 8 cm^2 area on the backs of the mice was shaved. Mice in group 1 received no further treatment and were used as controls. Mice in groups 2 and 3 were treated with a single dose of 300 µl of JP-8 and Jet-A, respectively. Mice in group 4 were used as positive controls and injected with CP. Two mice in each of the groups 2, 3 and 4 were sacrificed at 24 hour intervals, i.e., at 24, 48 and 72 hours after jet fuel treatment. Blood and bone marrow samples were collected and smears were prepared on clean microscopic slides. All smears were air-dried and the cells on the slides were fixed in absolute methanol. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C. Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium phosphate buffer, pH 7.4) before

microscopic examination. For each animal, 2000 consecutive PCE in blood and bone marrow were examined to determine the incidence of MN. The results were decoded after complete microscopic evaluation.

Results: incidence of MN/2000 PCE presented in Table H-2 indicated a gradual and time-related increase in the frequency of MN in both blood and bone marrow of mice exposed JP-8 and Jet-A, as compared with those in control mice. The incidence of MN was significantly different between jet fuel-exposed and control mice at 72 hours in the blood and at 48 hours in the bone marrow. Positive control mice injected with CP also exhibited similar increase in the incidence of MN.

Table H-2 (Experiment 2 in Houston): Incidence of micronuclei (MN in PCE) in the blood and bone marrow of mice exposed to 300 µl JP-8 or Jet-A (dermal).

Group	# Mice Studied	Sacrifice Hour	Total PCE Examined	Individual Mouse Data MN/2,000 PCE		Group Mean MN in 2,000 PCE
BLOOD - PCE						
Control	2		4,000	4	4	4.0
JP-8	2	24	4,000	6	6	6.0
JP-8	2	48	4,000	7	8	7.5
JP-8	2	72	4,000	12	10	11.0
Jet-A	2	24	4,000	5	6	5.5
Jet-A	2	48	4,000	6	7	6.5
Jet-A	2	72	4,000	8	9	8.5
CP	2	24	4,000	19	23	21.0
CP	2	48	4,000	34	42	38.0
CP	2	72	4,000	22	26	24.0
BONE MARROW - PCE						
Control	2		4,000	6	4	5.0
JP-8	2	24	4,000	9	8	8.5
JP-8	2	48	4,000	15	10	12.5
JP-8	2	72	4,000	9	8	8.5
Jet-A	2	24	4,000	8	7	7.5
Jet-A	2	48	4,000	13	10	11.5
Jet-A	2	72	4,000	9	7	8.0
CP	2	24	4,000	31	42	36.5
CP	2	48	4,000	74	57	65.5
CP	2	72	4,000	28	36	32.0

Blood and bone marrow were collected when the mice were sacrificed at 24, 48 and 72 h after JP-8 or Jet-A treatment.
In each mouse, 2000 consecutive PCE in the blood and bone marrow were examined.
CP (cyclophosphamide): 40 mg/kg bodyweight, intraperitoneal injection.

Experiment #3: Exposure-Time-Course Study

Aim: To determine the incidence of MN in the blood and bone marrow cells of mice exposed to 3 successive and increasing doses of JP-8 and Jet-A (dermal) – Exposure-Time-Course evaluation of genotoxicity in PCE.

The observations in earlier experiments 1 and 2 have indicated an increased incidence of MN in both blood and bone marrow cells of mice treated on the skin with JP-8 and Jet-A. A comprehensive investigation was carried out to determine whether the blood and bone marrow cells from mice exposed to JP-8 and Jet-A, indeed, exhibit a time-related increase in MN. In this

investigation, in addition to Dr. Vijayalaxmi (Principal Investigator), Dr. Andrew Kligerman at the Environmental Carcinogenesis Division, U.S. Environmental Protection Agency (EPA), Research Triangle Park, NC, has examined a complete and duplicate set of 'coded' microscope slides prepared from the blood and bone marrow smears from these mice. This step was initiated to obtain an independent evaluation and confirmation of the observations.

The protocol used for the experiment was as follows. A total of 40 adult, 8 to 10 weeks old, C3H/HeN (MTV-) female mice were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls, shave only - 10 mice
Group 2: JP-8, 300 µl applied on the skin on Monday - 10 mice
Group 3: Jet-A, 300 µl applied on the skin on Monday - 10 mice
Group 4: CP-40 mg/kg body weight (positive controls) - 10 mice
Blood smears were prepared on Mon, Tues, Wed' & Thursday (0, 24, 48 & 72 hour after the treatment)
Bone marrow smears were prepared on Thursday (72 hour after the treatment)

Two hours before the start of the experiment, an approximately 8 cm^2 area on the backs of the mice was shaved. Mice in group 1 received no further treatment and were used as controls. Mice in groups 2 and 3 were treated with a single dose of 300 µl of JP-8 and Jet-A, respectively. Mice in group 4 were used as positive controls and were injected with CP. Small drops of blood were collected by snipping the tails of all mice at the start of the experiment (t = 0), and at 24, 48 and 72 hours following treatments. The blood was immediately placed on clean microscope slides to prepare smears. Bone marrow was collected when all mice were sacrificed at 72 hours following the treatment and smears were prepared on clean microscope slides. All smears were air-dried and the cells on the slides were fixed in absolute methanol. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C. Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium phosphate buffer, pH 7.4) before microscopic examination. One complete set was mailed to Dr. Andrew Kligerman at EPA for microscopic examination while the second set was evaluated at UTHSC. Thus, two independent investigators (Andy at EPA and Vijay at UTHSC) assessed the incidence of MN in peripheral blood and bone marrow cells. Each investigator used a fluorescence microscope fitted with appropriate filters for acridine orange stain. For each animal, 2000 consecutive PCE in blood and bone marrow were examined to determine the incidence of MN. The results were decoded after complete microscopic evaluation by both investigators. The results from both investigators were subjected to statistical analyses.

Results: The incidence of MN/2000 PCE in the blood and bone marrow recorded by individual investigator as well as the combined data are presented in **Table H-3**. Both investigators, Andy and Vijay, have documented an increase in the frequency MN/2000 PCE in the blood of mice treated with JP-8 and Jet-A over the 72 hour period, as compared with those in control mice. The difference between control animals and mice treated with jet fuels was statistically significant at 72 hours. Similarly, in the bone marrow, a higher incidence of MN was documented in JP-8 and Jet-A treated mice, as compared with controls. The positive control mice injected CP also showed the expected and significant increases in MN in both tissues. Thus, at the dose tested, dermal exposure of JP-8 and Jet-A resulted in a small but significant increase in genotoxicity in hematopoietic cells of mice.

Table H-3 (Experiment 3 in Houston): Incidence of micronuclei (MN in PCE) in the blood and bone marrow of mice Exposed to 300 ml JP-8 or Jet-A (dermal).

Group	# Mice Studied	Sacrifice Hour	Investigator	Total PCE Examined	Mean MN/2,000 PCE	Group Mean MN in 2,000 PCE	Group Std. Dev.
BLOOD – PCE							
Control	10	0	Andy	20,000	4.8		
Control	10	0	Vijay	20,000	4.5	4.7	1.8
Control	10	24	Andy	20,000	6.4		
Control	10	24	Vijay	20,000	4.4	5.4	2.3
Control	10	48	Andy	20,000	8.3		
Control	10	48	Vijay	20,000	4.5	6.4	3.1
Control	10	72	Andy	20,000	4.3		
Control	10	72	Vijay	20,000	4.4	4.4	3.0
JP-8	10	0	Andy	20,000	4.4		
JP-8	10	0	Vijay	20,000	4.1	4.3	1.9
JP-8	10	24	Andy	20,000	5.5		
JP-8	10	24	Vijay	20,000	4.8	5.2	2.1
JP-8	10	48	Andy	20,000	8.4		
JP-8	10	48	Vijay	20,000	6.1	7.3	2.7
JP-8	10	72	Andy	20,000	9.1		
JP-8	10	72	Vijay	20,000	6.9	8.0	3.9
Jet-A	10	0	Andy	20,000	4.9		
Jet-A	10	0	Vijay	20,000	4.2	4.6	1.8
Jet-A	10	24	Andy	20,000	5.1		
Jet-A	10	24	Vijay	20,000	4.4	4.8	1.9
Jet-A	10	48	Andy	20,000	9.7		
Jet-A	10	48	Vijay	20,000	6.4	8.1	3.3
Jet-A	10	72	Andy	20,000	8.6		
Jet-A	10	72	Vijay	20,000	7.8	8.2	3.6
CP	10	0	Andy	20,000	5.2		
CP	10	0	Vijay	20,000	4.5	4.9	2.3
CP	10	24	Andy	20,000	13.5		
CP	10	24	Vijay	20,000	14.4	14.0	5.5
CP	10	48	Andy	20,000	40.1		
CP	10	48	Vijay	20,000	29.6	34.9	10.4
CP	10	72	Andy	20,000	13.3		
CP	10	72	Vijay	20,000	15.7	14.5	3.6
BONE MARROW – PCE							
Control	10	72	Andy	20,000	5.6		
Control	10	72	Vijay	20,000	5.1	5.4	2.7
JP-8	10	72	Andy	20,000	6.0		
JP-8	10	72	Vijay	20,000	6.8	6.4	2.7
Jet A	10	72	Andy	20,000	8.0		
Jet A	10	72	Vijay	20,000	8.3	8.2	3.5
CP	10	72	Andy	20,000	8.9		
CP	10	72	Vijay	20,000	9.7	9.3	3.3

Blood was collected before (0) and at 24, 48 and 72 hours after JP-8 or Jet-A treatment.
Bone marrow was collected when the mice were sacrificed at 72 hours after JP-8 or Jet-A treatment.
In each mouse, 2000 consecutive PCE in the blood were examined.
CP (cyclophosphamide): 40 mg/kg bodyweight, intraperitoneal injection.

The data were published - Vijayalaxmi, AD. Kligerman, TJ. Prihoda and SE. Ullrich. Cytogenetic studies in mice treated with jet fuels, Jet-A and JP-8. Cytogenetics and Genome Research. 104, 371-375, 2004. (pdf. file is attached).

Experiment #4: Dose Escalation Study

Aim: To determine the incidence of MN in the blood and bone marrow cells of mice exposed to 3 successive and increasing doses of JP-8 and Jet-A (dermal) – Evaluation of genotoxicity in both PCE and NCE.

The data reported in an experiment #3 (Vijayalaxmi et al., Cytogenet Genome Res., 104, 371-375, 2004) indicated a small but significant increase in MN in the blood and bone marrow cells of mice treated with JP-8 and Jet-A. A detailed investigation was carried out to extend these observations using the classical three-dose regimen protocol, to determine whether a dose-related increase in MN will be observed in mice which were treated with 3 successive and increasing doses of JP-8 and Jet-A. The slides prepared from this experiment were examined by Dr. Vijayalaxmi (Principal Investigator) and Dr. Andrew Kligerman at EPA. This is to obtain an independent evaluation and confirmation of the observations.

The protocol used for the experiment was as follows. A total of 100 adult, 8 to 10 weeks old, C3H/HeN (MTV-) female mice were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls, shave only, for JP-8 -10 mice.
Group 2: Controls, shave only, for Jet-A - 10 mice.
Group 3: JP-8, 300 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x300 µl) -10 mice.
Group 4: JP-8, 100 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x100µl) -10 mice.
Group 5: JP-8, 50 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x50 µl) -10 mice.
Group 6: Jet-A, 300 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x300 µl) -10 mice.
Group 7: Jet-A, 100 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x100 µl) -10 mice.
Group 8: Jet-A, 50 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x50 µl) -10 mice.
Group 9: CP-40 mg/kg body weight (positive controls for JP-8) -10 mice.
Group 10: CP-40 mg/kg body weight (positive controls for Jet-A) -10 mice.

Blood smears were prepared at 24 hours after the 3rd application (Thursday).
Bone marrow smears were prepared at 24 hours after the 3rd application (Thursday).

Two hours before the start of the experiment, an approximately 8 cm^2 area on the backs of the mice was shaved. Mice in groups 1 and 2 received no further treatment and were used as controls. Mice in groups 3-5 were treated with 3 successive and increasing doses JP-8 (Monday, Tuesday and Wednesday). Mice in groups 6-8 were treated with 3 successive and increasing doses Jet-A (Monday, Tuesday and Wednesday). Mice in groups 9 and 10 were used as positive controls and were injected with CP. All mice were sacrificed on Thursday, 24 hours following the last treatment. Blood and bone marrow samples were collected and smears were prepared on clean microscopic slides. All smears were air-dried and the cells on the slides were fixed in absolute methanol. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C. Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium

phosphate buffer, pH 7.4) before microscopic examination. One complete set was mailed to Dr. Andrew Kligerman at EPA for microscopic examination while the second set was evaluated at UTHSC. Thus, two independent investigators (Andy at EPA and Vijay at UTHSC) assessed the incidence of MN in peripheral blood and bone marrow cells. Each investigator used a fluorescence microscope fitted with appropriate filters for acridine orange stain. For each animal, 2000 consecutive PCE in blood and bone marrow were examined to determine the incidence of MN. The results were decoded after complete microscopic evaluation by both investigators. The results from both investigators were subjected to statistical analyses.

Results: The incidence of MN/2000 PCE in the blood and bone marrow cells recorded by individual investigator as well as the combined data are presented in **Table H-4a**. Neither investigator observed a significant and dose-dependent increase in MN in mice treated with JP-8 and Jet-A, as compared with those in control mice. The positive control mice injected CP showed the expected significant increases in MN in both tissues. Indeed, the results of this experiment #4 are contrary to general expectation that the incidence of MN correlates with the dose of 'genotoxic' agents.

During the discussion in the 2004 annual meeting in Tucson, a suggestion was made that the frequency of MN should also be evaluated in the NCE of blood and bone marrow tissues. Vijay has examined 10,000 consecutive NCE in blood and 2000 consecutive NCE in bone marrow. The incidence of MN/10000 NCE in blood and MN/2000 NCE of bone marrow are presented in **Table H-4b**. In the NCE also, no significant and dose-dependent increase in MN was observed in mice treated with JP-8 and Jet-A, as compared with those in control mice.

Thus, the increased MN reported in mice treated with a single dose of JP-8 and Jet-A (experiment #3, Vijayalaxmi et al., Cytogenet Genome Res., 104, 371-375, 2004) could not be extended to the 3 successive and increasing doses of JP-8 and Jet-A.

Skin tissues were collected from all mice when they were sacrificed and fixed in Omnifix solution. Fixed tissues were sent to UTHSC where they were embedded in paraffin blocks. Thin sections (4-microns) were prepared on microscope slides. Coded slides were evaluated for DNA (8-hydroxyguanosine) and protein adducts, and apoptosis. No significant differences were observed between control animals and mice treated with JP-8 and Jet-A.

Table H-4a (Experiment 4 in Houston): Incidence of micronuclei (MN in PCE) in the blood and bone marrow of mice exposed to three successive and increasing doses of JP-8 or Jet-A (dermal).

Group	# Mice Studied	Investigator	Total PCE Examined	Mean MN/2,000 PCE	Group Mean MN in 2,000 PCE	Group Std. Dev.
JP-8: BLOOD - PCE						
Control	10	Andy	20,000	4.8	4.4	2.8
Control	10	Vijay	20,000	4.0		
3 x 50 µl	10	Andy	20,000	4.9	4.8	1.8
3 x 50 µl	10	Vijay	20,000	4.7		
3 x 100 µl	10	Andy	20,000	4.6	4.6	1.6
3 x 100 µl	10	Vijay	20,000	4.6		
3 x 300 µl	10	Andy	20,000	4.4	5.0	1.9
3 x 300 µl	10	Vijay	20,000	5.6		
CP	10	Andy	20,000	32.4	28.0	8.1
CP	10	Vijay	20,000	23.6		
Jet-A: BLOOD – PCE						
Control	10	Andy	20,000	4.3	4.3	2.3
Control	10	Vijay	20,000	4.2		
3 x 50 µl	10	Andy	20,000	4.9	5.4	2.6
3 x 50 µl	10	Vijay	20,000	5.9		
3 x 100 µl	10	Andy	20,000	3.6	4.6	2.0
3 x 100 µl	10	Vijay	20,000	5.5		
3 x 300 µl	10	Andy	20,000	3.7	4.8	2.1
3 x 300 µl	10	Vijay	20,000	5.9		
CP	10	Andy	20,000	25.8	26.4	4.4
CP	10	Vijay	20,000	27.0		
JP-8: BONE MARROW - PCE						
Control	10	Andy	20,000	5.1	5.1	2.2
Control	10	Vijay	20,000	5.1		
3 x 50 µl	10	Andy	20,000	3.9	4.2	1.9
3 x 50 µl	10	Vijay	20,000	4.5		
3 x 100 µl	10	Andy	20,000	4.4	4.6	1.6
3 x 100 µl	10	Vijay	20,000	4.7		
3 x 300 µl	10	Andy	20,000	4.3	4.8	1.6
3 x 300 µl	10	Vijay	20,000	5.3		
CP	10	Andy	20,000	29.5	35.2	10.9
CP	10	Vijay	20,000	20.8		
Jet-A: BONE MARROW – PCE						
Control	10	Andy	20,000	4.2	4.5	1.7
Control	10	Vijay	20,000	4.7		
3 x 50 µl	10	Andy	20,000	3.1	3.9	3.4
3 x 50 µl	10	Vijay	20,000	4.7		
3 x 100 µl	10	Andy	20,000	3.6	4.2	1.5
3 x 100 µl	10	Vijay	20,000	4.8		
3 x 300 µl	10	Andy	20,000	3.2	3.9	1.6
3 x 300 µl	10	Vijay	20,000	4.6		
CP	10	Andy	20,000	27.6	22.9	10.6
CP	10	Vijay	20,000	18.1		

Mice were treated with JP-8 or Jet-A on Monday, Tuesday and Wednesday.
Blood was collected when the mice were sacrificed on Thursday, i.e., 24 hours after the last treatment.
Bone marrow was collected when the mice were sacrificed on Thursday, i.e., 24 hours after the last treatment.
In each mouse, 2000 consecutive PCE in the blood were examined.
CP (cyclophosphamide): 40 mg/kg bodyweight, intraperitoneal injection.

Table H-4b (Experiment 4 in Houston): Incidence of micronuclei (MN in NCE) in the blood and bone marrow of mice exposed to three successive and increasing doses of JP-8 or Jet-A (dermal).

Group	# Mice Studied	Investigator	Total NCE Examined	Mean MN in 10,000 NCE	Group Mean Std. Dev.
JP-8: BLOOD - NCE					
Control	10	Vijay	100,000	2.4	1.3
3 x 50 µl	10	Vijay	100,000	2.3	0.9
3 x 100 µl	10	Vijay	100,000	2.2	1.1
3 x 300 µl	10	Vijay	100,000	2.3	1.1
CP	10	Vijay	100,000	13.4	2.5
Jet-A: BLOOD - NCE					
Control	10	Vijay	100,000	2.3	1.2
3 x 50 µl	10	Vijay	100,000	2.7	0.9
3 x 100 µl	10	Vijay	100,000	2.7	1.1
3 x 300 µl	10	Vijay	100,000	2.2	0.9
CP	10	Vijay	100,000	14.0	2.4
JP-8: BONE MARROW – NCE					
Control	10	Vijay	20,000	2.4	1.1
3 x 50 µl	10	Vijay	20,000	2.4	1.3
3 x 100 µl	10	Vijay	20,000	2.4	1.4
3 x 300 µl	10	Vijay	20,000	2.5	1.4
CP	10	Vijay	20,000	11.5	1.7
Jet-A: BONE MARROW – NCE					
Control	10	Vijay	20,000	2.5	1.4
3 x 50 µl	10	Vijay	20,000	2.3	0.7
3 x 100 µl	10	Vijay	20,000	2.8	0.8
3 x 300 µl	10	Vijay	20,000	2.7	1.2
CP	10	Vijay	20,000	9.6	2.0

Mice were treated with JP-8 or Jet-A on Monday, Tuesday and Wednesday.
Blood and bone marrow were collected when the mice were sacrificed on Thursday, i.e., 24 hours after the last treatment.
In each mouse, 10000 consecutive NCE in the blood and 2000 consecutive NCE in bone marrow were examined.
CP (cyclophosphamide): 40 mg/kg bodyweight, intraperitoneal injection.

Experiment #5: Repeat Study

Aim: To determine the incidence of MN in the blood and bone marrow cells of mice exposed to either a single or 3 successive and increasing doses of JP-8 and Jet-A (dermal) – A repeat investigation of genotoxicity in both PCE and NCE.

Since the results of the experiment #4 did not indicate JP-8 and Jet-A dose-dependent increase in MN, a repeat experiment #5 was conducted to determine whether the observations in earlier experiment #3 (single dose and increased MN) and experiment #4 (successive doses and no increased MN) be confirmed. Only blood smears were prepared for examination in this repeat experiment. The slides prepared from this experiment also were examined by Dr. Vijayalaxmi (Principal Investigator) and Dr. Andrew Kligerman at EPA. This is to obtain an independent evaluation and confirmation of the observations.

The protocol used for the experiment was as follows. A total of 60 adult, 8 to 10 weeks old, C3H/HeN (MTV-) female mice were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls, shave only -10 mice.
Group 2: JP-8, 300 µl applied on the skin on Monday -10 mice.
Blood smears were prepared on Thursday (72 hours after a single application, as in experiment #3).
Group 3: JP-8, 300 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x300 µl) -10 mice.
Blood smears were prepared on Thursday (24 hours after the 3rd application, as in experiment #4).
Group 4: Jet-A, 300 µl applied on the skin on Monday -10 mice.
Blood smears were prepared on Thursday (72 hours after a single application, as in experiment #3).
Group 5: Jet-A, 300 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x300µl) -10 mice.
Blood smears were prepared on Thursday (24 hours after the 3rd application, as in experiment #4).
Group 6: CP-40 mg/kg body weight (positive controls) -10 mice.

Two hours before the start of the experiment, an approximately 8 cm^2 area on the backs of the mice was shaved. Mice in group 1 received no further treatment and were used as controls. Mice in groups 2 and 4 were treated a single dose of JP-8 and Jet-A, respectively (Monday). Mice in groups 3 and 5 were treated with 3 successive 300µl doses JP-8 and Jet-A, respectively (Monday, Tuesday and Wednesday). Mice in group 6 were used as positive controls and were injected with CP. All mice were sacrificed on Thursday when the blood samples were collected to prepare smears on clean microscope slides, i.e., at 72 hours after the single dose (as in experiment #3) and at 24 hours after the 3 successive doses (as in experiment #4). All smears were air-dried and the cells on the slides were fixed in absolute methanol. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C. Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium phosphate buffer, pH 7.4) before microscopic examination. One complete set was mailed to Dr. Andrew Kligerman at EPA for microscopic examination while the second set was evaluated at UTHSC. Thus, two independent investigators (Andy at EPA and Vijay at UTHSC) assessed the incidence of MN in peripheral blood and bone marrow cells. Each investigator used a fluorescence microscope fitted with appropriate filters for acridine orange stain. For each animal, 2000 consecutive PCE in the blood were examined to determine the incidence of MN. The results were decoded after complete microscopic evaluation by both investigators. The results from both investigators were subjected to statistical analyses.

Results: The incidence of MN/2000 PCE in the blood recorded by individual investigator as well as the combined data is presented in **Table H-5**.Neither investigator observed a significant increase in MN in mice treated with either a single or 3 successive doses of JP-8 and Jet-A, as compared with those in control mice. Vijay has also examined 10,000 consecutive NCE in blood smears. No significant increase in MN was observed in NCE of mice treated with JP-8 and Jet-A, as compared with those in control mice (**Table H-5**).

Thus, the increased MN reported in mice treated with a single dose of JP-8 and Jet-A (experiment #3) could not be confirmed. However, the absence of an increase in MN in mice treated with 3 successive doses of JP-8 and Jet-A was, indeed, confirmed in this repeat investigation.

Table H-5 (Experiment 5 in Houston): Incidence of micronuclei (MN in **PCE/NCE**) in the blood of mice exposed to a single or three successive 300 µl of JP-8 or Jet-A (dermal).

Group	# Mice Studied	Investigator	Total PCE Examined	Mean MN/2,000 PCE	Group Mean MN in 2,000 PCE	Group Std. Dev.
BLOOD - PCE						
Control	10	Andy	20,000	5.1	4.4	1.8
Control	10	Vijay	20,000	3.7		
JP-8: 300 µl (*)	10	Andy	20,000	4.6	4.9	1.5
JP-8: 300 µl (*)	10	Vijay	20,000	5.1		
JP-8: 3 x 300 µl (**)	10	Andy	20,000	4.4	4.9	1.7
JP-8: 3 x 300 µl (**)	10	Vijay	20,000	5.4		
Jet-A: 300 µl (*)	10	Andy	20,000	5.4	5.8	1.5
Jet-A: 300 µl (*)	10	Vijay	20,000	6.1		
Jet-A: 3 x 300 µl (**)	10	Andy	20,000	6.0	6.5	2.4
Jet-A: 3 x 300 µl (**)	10	Vijay	20,000	7.0		
CP	10	Andy	20,000	38.7	31.1	9.9
CP	10	Vijay	20,000	23.4		
Group	# Mice Studied	Investigator	Total NCE Examined	Mean MN in 10,000 NCE		Group Std. Dev.
BLOOD - NCE						
Control	10	Vijay	100,000	2.1		1.2
JP-8: 300 ml (*)	10	Vijay	100,000	2.4		1.1
JP-8: 3 x 300 ml (**)	10	Vijay	100,000	2.1		1.2
Jet-A: 300 ml (*)	10	Vijay	100,000	2.5		1.1
Jet-A: 3 x 300 ml (**)	10	Vijay	100,000	2.6		1.2
CP	10	Vijay	100,000	13.4		2.1

(*) : Mice were treated with JP-8 or Jet-A on Monday. Blood was collected on Thursday, i.e., 72 hours after the treatment (as in experiment #3).

(**) : Mice were treated with JP-8 or Jet-A on Monday, Tuesday and Wednesday. Blood was collected on Thursday, i.e., 24 hours after the last treatment (as in experiment #4).

In each mouse, 2000 consecutive NCE in the blood were examined.

CP (cyclophosphamide): 40 mg/kg bodyweight, intraperitoneal injection.

Experiment #6: Hypothesis Testing Study

Aim: To determine the incidence of MN in the blood and bone marrow cells of mice exposed to JP-8, either 3 successive doses/week or 1 dose/week for 3 consecutive weeks (dermal) – Evaluation of genotoxicity in both PCE and NCE and testing of hypotheses.

The probable reason(s) for the absence of an increase in MN in mice treated with single or 3 successive and increasing doses of JP-8 and Jet-A (experiment #5) are not clear. The proposed hypotheses were: (1) 'adaptive response' induced by the first dose of JP-8 (which induced a very small increase in MN) which then conferred resistance to the second and third doses of JP-8, and/or (2) if the 'stem cells' in bone marrow of mice were extensively damaged by the first dose of JP-8, there would not have been sufficient time for the cells to regenerate and damaged by the second and third doses of JP-8. Experiment #6 was conducted to test these hypotheses. The slides prepared from this experiment also were examined by Dr. Vijayalaxmi (Principal Investigator) and Dr. Andrew Kligerman at EPA. This is to obtain an independent evaluation and confirmation of the observations.

The protocol used for the experiment was as follows. A total of 45 adult, 8 to 10 weeks old, C3H/HeN (MTV-) female mice were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls, shave only -10 mice. Monday of week 1.
Blood smears prepared at 48 hours after the shave (Wednesday of week 1).
Group 2: JP-8, 300 µl applied on the skin on Monday, Tuesday and Wednesday (total 3x300µl) -10 mice.
Blood smears were prepared on Friday (48 hours after the third application).
Group 3: JP-8, 300 µl applied 3 times at one week intervals (total 3x300µl) on the skin of the same 10 mice.
First application on Monday of week 1.
Blood smears were prepared on Wednesday of week 1 (48 hours after the first application).
Second application on Monday of week 2.
Blood smears were prepared on Wednesday of week 2 (48 hours after the second application).
Third application on Monday of week 3.
Blood smears were prepared on Wednesday of week 3 (48 hours after the third application)
Group 4: CP-40 mg/kg body weight (positive controls) -5 mice.

Two hours before the start of the experiment, an approximately 8 cm^2 area on the backs of the mice was shaved. Mice in group 1 received no further treatment and were used as controls. Mice in groups 2 and 3 were treated either 3 successive doses of JP-8 or one dose/week for 3 consecutive weeks. Mice in group 4 were used as positive controls and were injected with CP. Blood samples were collected on Wednesdays, i.e., 48 hours after JP-8 treatment, and smears were prepared on clean microscopic slides. All smears were air-dried and the cells on the slides were fixed in absolute methanol. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C. Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium phosphate buffer, pH 7.4) before microscopic examination. One complete set was mailed to Dr. Andrew Kligerman at EPA for microscopic examination while the second set was evaluated at UTHSC. Thus, two independent investigators (Andy at EPA and Vijay at UTHSC) assessed the incidence of MN in peripheral blood and bone marrow cells. Each investigator used a fluorescence microscope fitted with appropriate filters for acridine orange stain. For each animal, 2000 consecutive PCE in the blood were examined to determine the incidence of MN. The results were decoded after complete microscopic evaluation by both investigators. The results from both investigators were subjected to statistical analyses

Results: The incidence of MN/2000 PCE in the blood recorded by individual investigator as well as the combined data is presented in **Table H-6**.Neither investigator observed a significant increase in MN in mice exposed to JP-8, either 3 successive doses/week or 1 dose/week for 3 consecutive weeks, as compared with those in control mice. Vijay has examined 10,000 consecutive NCE in blood smears. . No significant increase in MN in NCE was also observed in mice treated with either a single or 3 successive doses of JP-8 and Jet-A, as compared with those in control mice (**Table H6**).

Table H-6 (Experiment 6 in Houston): Incidence of micronuclei (MN in **PCE/NCE**) in the blood of mice exposed to JP-8, three successive 300 µl/week or 300 µl/day/week for 3 weeks (dermal).

Group	# Mice Studied	Investigator	Total PCE Examined	Mean MN/2,000 PCE	Group Mean MN in 2,000 PCE	Group Std. Dev.
BLOOD - PCE						
Control	10	Andy	20,000	4.0	4.0	2.0
Control	10	Vijay	20,000	3.9		
JP-8: 3 x 300 µl (*)	10	Andy	20,000	5.1	5.3	2.5
JP-8: 3 x 300 µl (*)	10	Vijay	20,000	5.4		
JP-8: 300 µl-Week1 (**)	10	Andy	20,000	5.3	5.4	2.0
JP-8: 300 µl-Week1 (**)	10	Vijay	20,000	5.4		
JP-8: 300 µl-Week2 (**)	10	Andy	20,000	4.4	4.5	1.9
JP-8: 300 µl-Week2 (**)	10	Vijay	20,000	4.6		
JP-8: 300 µl-Week3 (**)	10	Andy	20,000	4.3	4.4	1.8
JP-8: 300 µl-Week3 (**)	10	Vijay	20,000	4.4		
CP	5	Andy	10,000	37.2	18.6	7.0
CP	5	Vijay	10,000	34.8		
Group	# Mice Studied	Investigator	Total NCE Examined	Mean MN in 10,000 NCE		Group Std. Dev.
BLOOD - NCE						
Control	10	Vijay	100,000	1.8		0.9
JP-8: 3 x 300 µl (*)	10	Vijay	100,000	2.1		1.1
JP-8: 300 µl-Week1 (**)	10	Vijay	100,000	2.2		1.4
JP-8: 300 µl-Week2 (**)	10	Vijay	100,000	2.0		1.5
JP-8: 300 µl-Week3 (**)	10	Vijay	100,000	1.9		1.4
CP	5	Vijay	100,000	16.8		3.3

(*) : Mice were treated with JP-8 on Monday, Tuesday and Wednesday. Blood was collected on Friday, the last treatment, i.e., 48 hours after each treatment.

(**) : Mice were treated with JP-8 on each Monday of week 1, 2 and 3. Blood was collected on each Wednesday of week 1, 2 and 3, i.e., 48 hours after each treatment.

In each mouse, 2000 consecutive PCE in the blood were examined.

CP (cyclophosphamide): 40 mg/kg bodyweight, intraperitoneal injection.

The data from several of the above studies were published - Vijayalaxmi, AD. Kligerman, TJ. Prihoda and SE. Ullrich. Micronucleus studies in the peripheral blood and bone marrow of mice treated with jet fuels, Jet-A and JP-8. Mutation Research. 608, 82-87, 2006. (pdf. file is attached).

INHALATION EXPOSURE

Collaboration Studies with Dr. Mark L. Witten in Tucson

Experiment #1: Preliminary Study 1

Aim: To determine the incidence of MN in the blood and bone marrow cells of mice exposed to JP-8 (inhalation) – Evaluation of genotoxicity in PCE.

The protocol used for the experiment is as follows. A total of 24 adult, 8 to 10 weeks old, Swiss-Webster male mice were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls kelp in the laboratory – 8 mice
Group 2: JP-8, average 50 mg/m^3, 1 hour/day for 7 consecutive days - 12 mice
Group 3: MMC - 1mg/kg body weight (positive controls) - 4 mice
Blood smears were prepared at 24 hours after the last exposure of JP-8.
Bone marrow smears were prepared at 24 after the last exposure of JP-8.

Mice in group 1, kept in the laboratory, were used as controls. Mice in groups 2 were exposed to aerosolized JP-8 for 1 hour/day for 7 consecutive days. Mice in group 3 were used as positive controls and were injected with MMC. All mice were sacrificed at 24 hours following the last exposure. Blood and bone marrow samples were collected and smears were prepared on clean microscopic slides. All smears were air-dried and the cells on the slides were fixed in absolute methanol. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C. Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium phosphate buffer, pH 7.4) before microscopic examination. For each animal, 2000 consecutive PCE in blood and bone marrow were examined to determine the incidence of MN. The results were decoded after complete microscopic evaluation.

Results: The incidence of MN/2000 PCE presented in **Table T-1a** indicated a small increase in MN in both blood and bone marrow tissues of mice exposed to JP-8 as compared with those in control mice. Positive control mice injected with MMC exhibited the expected increase in the incidence of MN.

Liver and spleens were collected from all mice when the animals were sacrificed to determine if JP-8 exposure had any influence on these organ weights. Both testes were also collected and fixed in Omnifix solution and sent to UTHSC where they were embedded in paraffin blocks. Thin sections, 4-microns, were prepared on microscope slides, and coded slides were evaluated for the incidence of cells in apoptosis. The data presented in **Table T-1b** indicated no significant differences in the average weights of liver and spleen. However, the incidence of cells in apoptosis was significantly increased in the testis of mice exposed to JP-8, as compared with controls.

Table T-1a (Experiment 1 in Tucson): Incidence of micronuclei (MN in PCE) in the blood and bone marrow of mice exposed to 50 mg/m3 JP-8 for 1 hour/day for 7 consecutive days (inhalation).

Group	# Mice Studied	Total PCE Examined	Mean MN in 2,000 PCE	Group Std. Dev.
BLOOD				
Control	8	16,000	6.6	1.3
JP-8	12	24,000	14.3	2.8
MMC	4	8,000	26.3	4.3
BONE MARROW				
Control	8	16,000	8.0	1.3
JP-8	12	24,000	16.3	2.6
MMC	4	8,000	34.5	6.9

Blood and bone marrow were collected when the mice were sacrificed at 24 hours after the last treatment.
In each mouse, 2000 consecutive PCE in the blood and bone marrow were examined.
MMC (mitomycin C): 1 mg/kg body weight, intraperitoneal injection.

Table T-1b (Experiment 1 in Tucson): Body weights, liver and spleen weights, and the incidence of cells in apoptosis in the testis of mice exposed to 50 mg/m^3 JP-8 for 1 hour/day, 7 consecutive days (inhalation).

	Control (+/- SEM)	JP-8 (+/- SEM)	Statistical significance
Body weight (grams)	32.6 (1.66) (n=8)	30.3 (0.70) (n=11)	p=0.186
Liver weight (grams)			
Absolute weight	1.8 (0.07)	1.5 (0.03)	p=0.002
Percent body weight	5.5 (0.14) (n=8)	5.0 (0.13) (n=11)	p=0.028
Spleen weight (grams)			
Absolute weight	0.09 (0.01)	0.11 (0.19)	p=0.767
Percent body weight	0.32 (0.03) (n=8)	0.42 (0.08) n=11)	p=0.272
Testis:			
Cells in apoptosis (in 100 tubules examined)	21.4 (1.28) (n=8)	26.5 (1.11) (n=12)	p=0.008

Tissues were collected when the mice were sacrificed at 24 hours after the last treatment.

Experiment #2: Preliminary study 2

Aim: To determine the incidence of MN in the blood and bone marrow cells of mice exposed to JP-8 (inhalation) – Evaluation of genotoxicity in PCE.

The results from the first preliminary experiment have indicated an increased incidence of micronuclei (MN) in both the blood and bone marrow cells of mice exposed to JP-8 through inhalation route. A second repeat experiment was conducted in mice exposed to small increases in JP-8 (50 and 70 mg/cubic meter) to see if there was a dose-related increase in MN.

The protocol used for the experiment is as follows. A total of 24 adult, 8 to 10 weeks old, Swiss-Webster male mice were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls kelp in the laboratory – 8 mice
Group 2: JP-8, average 50 mg/m^3, 1 hour/day for 7 consecutive days - 8 mice
Group 3: JP-8, average 70 mg/m^3, 1 hour/day for 7 consecutive days - 7 mice
Group 4: MMC - 1mg/kg body weight (positive controls) - 4 mice
Blood smears were prepared at 24 hours after the last exposure of JP-8.
Bone marrow smears were prepared at 24 after the last exposure of JP-8.

Mice in group 1, kept in the laboratory, were used as controls. Mice in groups 2 and 3 were exposed to aerosolized JP-8, 50 mg/m^3 and 70 mg/m^3, respectively, for 1 hour/day for 7 consecutive days. Mice in group 4 were used as positive controls and were injected with MMC. All mice were sacrificed at 24 hours following the last exposure. Blood and bone marrow samples were collected and smears were prepared on clean microscopic slides. All smears were air-dried and the cells on the slides were fixed in absolute methanol. All slides were air-dried again, and stored in a box kept in a dark place, at room temperature or at 4°C. Each slide was labeled with a random number which will not give the identity of the treatment group during microscopic evaluations. Coded slides were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. All slides were stained with acridine orange (0.01 mg/ml in 0.2 M sodium phosphate buffer, pH 7.4) before microscopic examination. For each animal, 2000 consecutive PCE in blood and bone marrow were examined to determine the incidence of MN. The results were decoded after complete microscopic evaluation.

Results: **Results**: The incidence of MN/2000 PCE presented in **Table T-2a**. Indicated a small increase in MN in both blood and bone marrow tissues of mice exposed to JP-8 as compared with those in control mice. However, the results did not indicate a clear JP-8-dose-dependent increase in the incidence of MN. Positive control mice injected with MMC exhibited the expected increase in the incidence of MN.

Liver and spleens were collected from all mice when the animals were sacrificed to determine if JP-8 exposure had any influence on these organ weights. Both testes were also collected and fixed in Omnifix solution and sent to UTHSC where they were embedded in paraffin blocks. Thin sections, 4-microns, were prepared on microscope slides, and coded slides were evaluated for the incidence of cells in apoptosis. The data presented in **Table T-2b** indicated no significant differences in the average weights of liver and spleen as well as in the incidence of cells in apoptosis in the testis of mice exposed to JP-8, as compared with controls.

General conclusions from the data from both the two experiments:

(1) The mean body weight of mice treated with JP-8 was significantly lower in the experiment 1 (older mice, ($p<0.01$) (Figure 1) as compared with the weights of the mice in experiment 2.
(2) The mean liver weight of JP-8 treated mice, expressed as percent of body weight, was significantly less in experiment 1 (older mice, $p<0.01$), but not significantly lower in experiment 2 (younger mice) (Figure 2).
(3) Significantly higher incidence of splenomegaly was recorded in JP-8 treated mice in both experiments ($p<0.022$) (Figure 3).

There were some concerns regarding the inadequate dosimetry of JP-8 that was inhaled by the experimental mice and also the absence of inclusion of sham-exposed mice for accurate comparison of the data collected in earlier experiments. As soon as these issues are resolved the proposed inhalation exposure investigations will be initiated.

Table T-2a (Experiment 2 in Tucson): Incidence of micronuclei (MN in PCE and NCE) in the blood and bone marrow of mice exposed to JP-8 for 1 hour/day for 7 consecutive days (inhalation).

Group	# Mice Studied	Total PCE Examined	Mean MN in 2,000 PCE	Group Std. Dev.
BLOOD - PCE				
Control	8	16,000	4.5	1.6
JP-8 (50.4 mg/m3)	8	16,000	6.4	1.4
JP-8 (70.4 mg/m3)	7	14,000	6.3	1.7
MMC	4	8,000	11.5	1.0
BONE MARROW - PCE				
Control	8	16,000	5.8	1.6
JP-8 (50.4 mg/m3)	8	16,000	9.3	2.1
JP-8 (70.4 mg/m3)	5	10,000	6.4	2.1
MMC	4	8,000	15.0	3.4
Group	# Mice Studied	Total NCE Examined	Mean MN in 2,000 NCE	Group Std. Dev.
BLOOD - NCE				
Control	8	16,000	3.3	1.0
JP-8 (50.4 mg/m3)	8	16,000	2.8	1.3
JP-8 (70.4 mg/m3)	7	14,000	2.7	1.1
MMC	4	8,000	7.8	1.7
BONE MARROW - NCE				
Control	8	16,000	3.3	1.0
JP-8 (50.4 mg/m3)	8	16,000	3.8	0.9
JP-8 (70.4 mg/m3)	5	10,000	2.9	1.9
MMC	4	8,000	10.3	1.7

Blood and bone marrow were collected when the mice were sacrificed at 24 hours after the last treatment.
In each mouse, 2000 consecutive PCE and 2000 consecutive NCE in the blood and bone marrow were examined.
MMC (mitomycin C): 1 mg/kg body weight, intraperitoneal injection.

Table T-2b (Experiment 1 in Tucson): Body weights, liver and spleen weights, and the incidence of cells in apoptosis in the testis of mice exposed to 50 mg/m^3 and 70.4 mg/m^3 JP-8 for 1 hour/day, 7 consecutive days (inhalation).

	Control (+/- SEM)	JP-8 (50.4 mg/m^3) (+/- SEM)	JP-8 (70.4 mg/m^3) (+/- SEM)
Body weight (grams)	24.8 (0.5) (n=8)	23.0 (0.4) (n=8)	22.0 (0.8) (n=7)
Liver weight (grams)			
Absolute weight	1.1 (0.05)	1.0 (0.06)	1.0 (0.05)
Percent body weight	5.5 (0.14) (n=8)	5.0 (0.13) (n=8)	5.0 (0.13) (n=7)
Spleen weight (grams)			
Absolute weight	0.1 (0.01)	0.1 (0.02)	0.08 (0.04)
Percent body weight	0.32 (0.03) (n=8)	0.42 (0.08) (n=8)	0.42 (0.08) (n=7)
Testis:			
Cells in apoptosis (in 100 tubules examined)	10.6 (1.03) (n=8)	11.4 (1.01) (n=8)	7.5 (1.33) (n=7)

Tissues were collected when the mice were sacrificed at 24 hours after the last treatment.

INHALATION - WHOLE BODY EXPOSURE

Collaboration Studies with Dr. Glenn Ritchie, WPAFB, OHIO

Experiment #1: Preliminary Study 1

Aim: To determine the genotoxic potential of JP-8 in the bone marrow cells of rats. The genotoxicity will be determined, using the classical MN assay in the bone marrow cells of mice, whole body exposed to JP-8 (inhalation route) - A preliminary study of genotoxiciy in PCE.

The protocol used for the experiment is as follows. A total of 80 adult rats were randomized to the following groups. The mean body weight of the animals in all groups was not significantly different.

Group 1: Controls kelp in the laboratory – 16 rats
Group 2: JP-8, average 0 mg/m^3 – 32 rats
Group 3: JP-8, average 250 mg/m^3 – 16 rats
Group 4: JP-8, average 500 mg/m^3 – 16 rats

JP-8 exposures were conducted in a THRU chamber (670 Liter capacity) for 6 hours (8 AM to 2 PM) each day, for a total of 90 days. All rats were sacrificed, randomly within 48 hours after the last exposure. Femur bones were collected and frozen femur bones were sent to Dr. Vijayalaxmi (principal investigator) at the University of Texas Health Science Center (UTHSC) in San Antonio, TX. The feasibility of using marrows isolated from frozen bone marrow samples for micronuclei investigations was undertaken. Unfortunately the marrow that was recovered from frozen bones was not suitable for MN evaluation.

HUMAN BLOOD LYMPHOCYTES

Collaboration Studies with Dr. David Harris, Tucson, AZ

Experiment #1: Preliminary Study 1

Aim: To determine the genotoxic potential of JP-8 in peripheral blood lymphocytes isolated from human volunteers exposed to jet fuel - A preliminary study of genotoxiciy evaluation in cultured blood lymphocytes.

Dr. David Harris had participated in immunological investigations using blood samples collected from approximately 60 human subjects: some of these subjects were exposed JP-8. Leukocytes separated from these blood samples are kept frozen at $-70^{o}C$ in his laboratory.

Experiments that were planned to determine the feasibility of using such frozen leukocytes for micronuclei investigations could not be under taken.

USAFOSR - UNPUBLISHED DATA FROM PREVIOUS INVESTIGATIONS

The unpublished data from earlier investigations under U. S. Air Force Office of Scientific Research grants - F49620-95-1-0337 for 2.45 GHz and F49620-98-1-0419 for 8.2 GHz) were published - "Cytogenetic Studies in Human Blood Lymphocytes Exposed *In Vitro* to 2.45 GHz or 8.2 GHz Radiofrequency Radiation. Radiation Research. 166, 532-538, 2006. (pdf. file is attached).

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